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Preparation of an Exonuclease from *Lactobacillus* Which Releases 5′-Mononucleotides from Polynucleotides*

Zulema J. Sabatini and Rollin D. Hotchkiss

ABSTRACT: A phosphodiesterase can be recovered from *Lactobacillus acidophilus* R26 cells which has the properties of an exonuclease with specificity for 5′-nucleotide esters. It hydrolyzes oligonucleotides of the ribo- and deoxyribonucleotide series, and *p*-nitrophenyl-5′-thymidine phosphate, to give exclusively 5′-mononucleotides. These substrates appear to compete for the same catalytic agent. Enzymatic activity is optimal at pH 8 with $MnCl_2$ at 5×10^{-4} M and in presence of a dithiol-stabilizing agent. Ca and Mg salts are also effective activators. High molecular deoxyribonucleic acid is degraded by the enzyme preparation with no evidence of intermediates other than mononucleotides. No evidence was obtained of activity on high molecular ribonucleic acid.

In a search for exonucleases capable of genetic variation, a study was undertaken in this laboratory of the nucleases of *Lactobacillus acidophilus* R26. The first enzyme found (R. D. Hotchkiss and H. G. Khorana, 1961, unpublished) was a diesterase hydrolyzing 3′-deoxyribonucleotide esters. This enzyme was then purified and shown to be an exonuclease hydrolyzing both ribo- and deoxyribooligonucleotides (Fiers and Khorana, 1963).

The present paper reports the discovery in *Lactobacillus* cells of an exonuclease of opposite polarity, *i.e.*, liberating 5′-ribo- and 5′-deoxyribomononucleotides. The substrate and cofactor requirements of partially purified preparations of the 5′-exonuclease are described.

Materials and Methods

Materials. Alkaline phosphatase, DNase I,[1] and RNA core were purchased from Worthington Biochemical Corp., Freehold, N. J. Cleland's reagent (dithiothreitol), *p*-nitrophenylthymidine 5′-phosphate (sodium salt), *p*-nitrophenol, and *p*-nitrophenyl phosphate (disodium salt, hexahydrate) were obtained from California Corp. for Biochemical Research, Los Angeles, Calif.

The following compounds (designated according to their 3′-phospho-5′ sequences) were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.: ApA,[1] CpA, CpU, ApG, ApC, GpC, and UpC. [*methyl*-^{3}H]Thymidine in H_2O (specific activity 3,47 Ci/mmole) was purchased from International Chemical and Nuclear Corp., City of Industry, Calif. Uridylate oligonucleotides and adenylate oligonucleotides were obtained from Miles Chemical Co., Elkhart, Ind.

DEAE-cellulose was Whatman microgranular DE32 from H. Reeve Angel, Clifton, N. J. Sephadex G-100, Sephadex G-15, and DEAE-Sephadex A-50 were from Pharmacia, Piscataway, N. J.

Preparation of Extracts. Bacterial strain *L. acidophilus* R26 was obtained from E. Travaglini of the Institute for Cancer Research, and also from the American Type Culture Collection (ATCC No. 314). The medium used for mass growth contained 40 g of Bacto Folic Acid Assay medium, 400 mg of ascorbic acid, 10 g of $NaAc \cdot 3H_2O$, 880 mg of $CaCl_2$, 6 g of casein hydrolysate (Nutritional Biochemicals, enzymatic), 400 mg of DL-alanine, 40 mg of L-tryptophan, 0.24 ml of glacial acetic acid, and 0.56 ml of Tween 80 per l. *L. acidophilus* was inoculated in the medium and grown at 37°; when cell density reached 5–10 $\times 10^8$ cells/ml, bacteria were

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[1] In this paper, nucleosides and nucleotides are represented by sequences in which A, C, G, U, represent the ribonucleoside residues adenosine, cytidine, etc., and p the phosphate residues starting from the left at the 5′ end. Deoxyriboside derivatives bear the d- prefix (except thymidylate). Other abbreviations are: PN-pT, *p*-nitrophenylthymidine 5′-phosphate; DNase I, pancreatic deoxyribonuclease; TDM 7.9, 0.05 M Tris-HCl buffer (pH 7.9) containing 0.075 M dithiothreitol and 10^{-3} M $MnCl_2$.

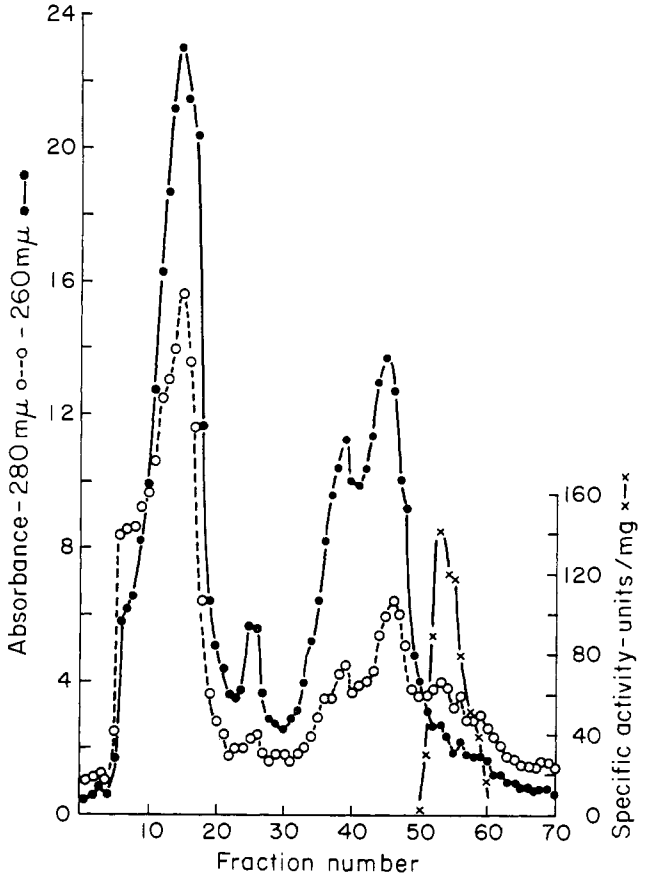

FIGURE 1: Chromatography of *L. acidophilus* enzyme on DEAE-cellulose. Elution at 0° in NaCl gradient (0–0.3 M); enzymatic activity toward PN-pT was observed only in fractions 50 to 60.

harvested in the centrifuge and washed once with cold 0.05 M Tris-HCl buffer (pH 7.9) containing 0.075 M dithiothreitol. The cells were frozen at −40° and disrupted by forcing through a narrow orifice under 20,000 psi in a Biotech Edebo Press (Edebo, 1960) from Biochemical Processes, Inc. Subsequent steps were done at 4°. The resulting paste was extracted twice in 40 ml, then in 15 ml, of 0.05 M Tris-HCl buffer (pH 8.5) containing 0.075 M dithiothreitol and 10^{-4} M $MnCl_2$, by suspending in a Virtis homogenizer then centrifuging at 12,000 rpm for 20 min. The combined supernatants were centrifuged at 100,000*g* for 2 hr and the pellet was discarded. Nucleic acids were precipitated from the supernatant with 0.4 ml of 1 M $MnCl_2$/40 ml and after preservation at −15° for 12 hr, the suspension was thawed and centrifuged for 40 min at 12,000 rpm. The supernatant containing the enzyme was loaded on a DEAE-cellulose column.

TABLE I: Recovery of Diesterase Activity During Purification.

Stage of Preparation	Protein (mg/ml)	Act. (Units/ mg of Protein)	Total Units	Recov (%)
Pooled supernatants	22.5	7	6930	
Supernatant of 100,000*g*	19	9	7110	100
$MnCl_2$ supernatant		9.5		
Pooled DEAE-cellulose fractions	1.78	95	3800	55
Pooled DEAE-Sephadex fractions	0.53	402	1300	19

TABLE II: Chromatographic Mobilities of Some Ribonucleosides and Ribonucleotides in Solvent 42.[a]

Dinucleoside Phosphate XpY	R_F Values of Derivatives				
		3′-Esterase Products		5′-Esterase Products	
	XpY	Xp	Y	X	pY
ApC	0.12	0.08[b]	0.36	0.47	0.04
ApG	0.09	0.08	0.50	0.47	0.03
ApA	0.14	0.08	0.47	0.47	0.06
CpC	0.06	0.06	0.36	0.36	0.04
CpU	0.07	0.06	0.46	0.36	0.05
CpA	0.07	0.06	0.47	0.36	0.06
UpC	0.06	0.08	0.36	0.46	0.04

[a] Measurements on Whatman No. 1 paper, descending, 15 hr in large jar, at 37°. (Small vessels, *e.g.*, plastic Chromatobox, in short runs gave somewhat higher R_F values at 37°.) [b] Cyclic nucleotides migrated like diesters: 2′,3′-cyclic AMP 0.30; 3′,5′-cyclic AMP 0.22; 2′,3′-cyclic CMP 0.15.

DEAE-cellulose Chromatography. The column, containing 5 g of DEAE-cellulose washed as recommended and equilibrated with Tris-HCl buffer (0.05 M, pH 7.5), containing 0.075 M dithiothreitol and 10^{-3} M $MnCl_2$, was loaded and washed with 100 ml of the same mixture, buffered at pH 7.9. Elution was performed at 0° with a gradient of NaCl increasing from 0 toward 0.3 M in the same solvent. Fractions of 4.5 ml each were collected and assayed as described. A typical elution pattern is represented in Figure 1. The change in relative absorption at 260 and 280 mμ serves as a useful empirical index of the active fractions. Fractions containing more than 10 units of enzymatic activity are pooled and dialyzed for 2 hr against 2 l. of TDM 7.9.

Protein was determined after precipitation with cold trichloroacetic acid by the method of Lowry as modified by Oyama and Eagle (1956). Whenever the process was interrupted at any step, the enzyme solutions were preserved in the frozen state, or for a few hours only, at 4°.

DEAE-Sephadex Chromatography. The dialyzed material was applied to a pretreated DEAE-Sephadex A-50 column (1 g of DEAE-Sephadex A-50, washed with 0.5 M NaCl, then with distilled water, and equilibrated with TDM 7.9) and washed with TDM 7.9. Elution was performed stepwise with 0.1 and 0.2 M NaCl in TDM 7.9. Enzyme activity is eluted in the second stage; 3-ml fractions were taken and tubes with activity were pooled and lyophilized. The activity proved to be somewhat labile and further purification was

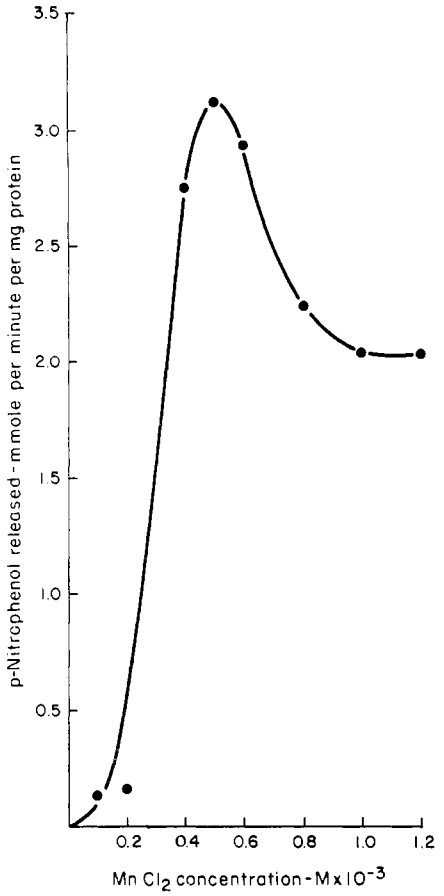

FIGURE 2: Activation of DEAE-Sephadex enzyme preparation by $MnCl_2$. The substrate PN-pT was used in the standard assay.

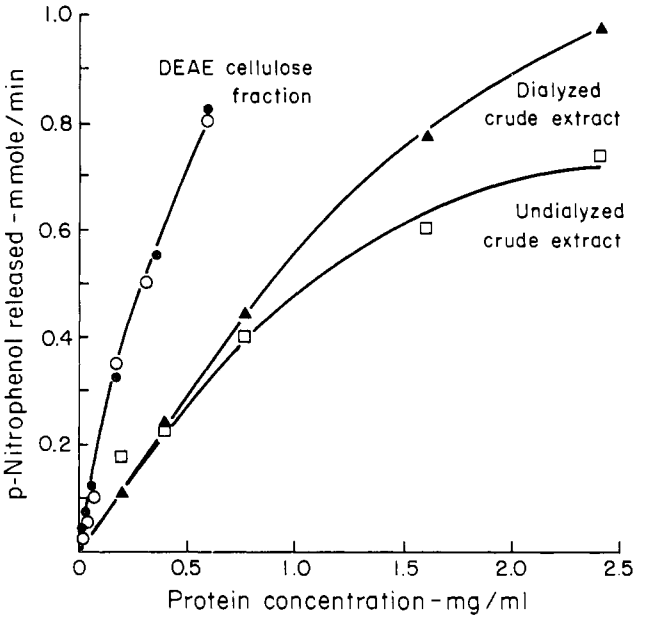

FIGURE 3: Concentration dependence and self-inhibition of several enzyme preparations. The standard assay with PN-pT was used. Two different DEAE-cellulose preparations (circles and filled circles) were fully comparable.

not attempted. A typical fractionation is summarized in Table I.

Measurement of Enzyme Activity. For enzyme assay, using the chromogenic substrate, *p*-nitrophenylthymidine 5′-phosphate, the standard mixture contains: 2 μmoles of PN-pT, 20 μmoles of Tris buffer (pH 7.9), 0.4 μmole of $MnCl_2$, 0.20 μmole of EDTA, 3 μmoles of dithiothreitol, and enzyme, in a total volume of 0.4 ml. Liberation of *p*-nitrophenol at 37° is followed by the optical density at 400 mμ, 50 μl of digest being diluted in 1 ml of 1 M Na_2CO_3–0.05 M EDTA. The molar extinction of *p*-nitrophenol at pH above 8.7 is taken to be 16,400 at 400 mμ.

The unit of enzymatic activity was defined as that amount which released 1 μmole of nitrophenol/ml in 30 min. Present preparations have given activities in the range of 200–500 units/mg of protein.

Hydrolysis of oligonucleotides was followed by quantitative paper chromatography of substrate and products using approximately 0.05-μmole quantities per assay. The standard assay mixture is like that for PN-pT except that about 0.4 μmole of substrate (ApC, etc.) is contained in the 0.4 ml of incubated mixture.

Paper Chromatographic Assay. Enzymatically treated substrates were analyzed with solvent 42 (see below) by descending chromatography on Whatman No. 1 paper at 37°. After chromatograms were developed, product and substrate spots were eluted in 0.01 N HCl and their concentrations were calculated from ultraviolet absorbance using either the wavelength of peak absorbance or 260 mμ.

Chromatographic solvent mixture 42 contains: *sec*-butyl alcohol–*n*-propyl alcohol–1 M ammonium acetate (pH 4.8) (5:3:3, v/v). It was devised for distinguishing several of the 5′- and 3′-mononucleotide pairs from each other as well as from the related nucleosides. The typical R_F values for prolonged chromatography, shown in Table II, somewhat minimize the true migration rates, but are selective enough to permit unambiguous identification of the products.

Enzyme Treatment of DNA. A culture of *Escherichia coli* 15T⁻ was labeled with [*methyl*-^{3}H]thymidine during growth and DNA was recovered according to the procedure of Lehman (1960). The DNA was purified by the chloroform–amyl alcohol and alcohol precipitations as used for pneumococcal-transforming DNA (Hotchkiss, 1966), followed by one phenol extraction and alcohol precipitation. It had a specific activity of 8.3 μCi/mg.

This DNA was treated with the *L. acidophilus* enzyme and its hydrolysis was followed by the appearance of acid-soluble material: 50-μl aliquots were precipitated with 0.2 ml of cold 10% trichloroacetic acid (containing 0.1% thymidine) using as carrier 0.1 ml of cold DNA (300 μg/ml) and 0.05 ml of bovine serum albumin (20 mg/ml); after centrifugation the radioactivity was measured in 0.2 ml of supernatant mixed with 10 ml of Bray's solution (Bray, 1960) using a Nuclear-Chicago liquid scintillation spectrometer.

For the run in Sephadex G-100 and G-15, the columns were 30 cm high and had a diameter of 1 cm, the solvent was 0.01 M NaCl, and the fraction size was 1 ml of which 0.2 ml was used to measure radioactivity. When pneumococcal DNA was used, optical density measurements were made following 0.25 N perchloric acid precipitation.

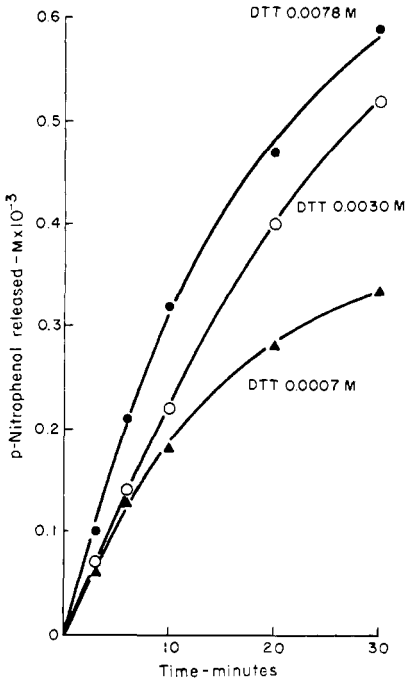

FIGURE 4: Stabilization of activity of DEAE-cellulose enzyme preparation by dithiothreitol during assay.

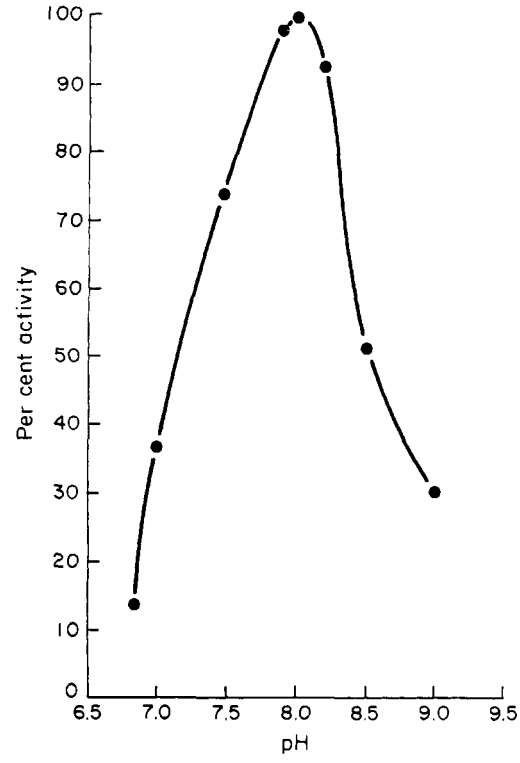

FIGURE 5: Enzyme activity at various pH values, as per cent of activity at maximum (pH 8.0). Buffering was done with Tris-HCl (0.05 M); other components as in standard assay.

Results

Properties of the Enzyme. The enzyme activity was studied under different conditions using *p*-nitrophenylthymidine 5′-phosphate as substrate. It has an absolute requirement for some divalent cations. Maximal activity was given by Mn^{2+} (Figure 2) or Ca^{2+} in concentrations from 4 to 6 $\times$ 10^{-4} M. At equivalent concentration Mg^{2+} gave only 35% of this maximal activity, leveling off at about 50% at higher concentration. In addition, in the first steps of purification EDTA (1 $\times$ 10^{-4} M) was necessary to yield maximal activity. There was no activation by Zn^{2+} or Co^{2+} salts. The activity is lost irreversibly when the enzyme preparation is dialyzed for 2 hr against buffer that does not contain Mn^{2+}. If EDTA is added to the reaction mixture without a cation, supplement activity is completely inhibited.

Some self-inhibition apparent at higher concentrations of the crude extract disappears on purification, and activity becomes essentially proportional to protein concentration after DEAE-cellulose fractionation (Figure 3).

After DEAE-Sephadex fractionation, EDTA no longer increases activity, and $MnCl_2$ gives maximal activation at levels approximately proportional to protein concentration (about 5 $\times$ 10^{-3} mmole/100 μg of total protein at this stage). These results are consistent with the possibility that the enzyme is activated, and also protected from irreversible inactivation, by the presence of Mn^{2+} and certain other ions. There are some indications from elution and ultrafiltration that absence of Mn^{2+} leads to aggregation.

The enzyme is stabilized in the reaction mixture by the addition of dithiothreitol (Figure 4). The pH optimum of the reaction is about 8.0 (Figure 5). Activities at 25 and 55° are about 58 and 16%, respectively, of that at 37°.

On chromogenic substrate PN-pT the K_m was about 6 $\times$ 10^{-3} M and a typical preparation gave V_{max} = 1660 μmoles/hr per mg of protein. Phosphomonoesterase activity (toward nucleotides or nitrophenyl phosphate) could not be detected in the preparations.

Activity on Oligo- and Polyribonucleotides. A series of ribodinucleoside phosphates, ApG, CpU, ApA, ApC, and UpC, were treated with *L. acidophilus* enzyme. In each case cleavage released a nucleoside plus a nucleotide with phosphorus in the 5′ position (thus ApC releases adenosine, A, and 5′-cytidylic acid, pC). The cleavage of the ribonucleotides was followed by paper chromatography using solvent 42 at 37°, which in a run of 18 hr allowed a good separation between the dinucleoside phosphate and the 5′-mononucleotide. In general this procedure also allowed identification of both the nucleoside and the 5′-mononucleotide produced, demonstrating that 3′-nucleotides were not formed. The ultraviolet-absorbing spots that were detected were quantitatively eluted

TABLE III: Activity of DEAE-cellulose-Purified Enzyme Preparations on Various Ribodinucleoside Phosphates.

Substrate	K_m (M)	V_{max} (μmoles/hr per mg)	Products
ApG	2.5 $\times$ 10^{-4}	50,000	A + pG
ApA	4.3 $\times$ 10^{-4}	120,000	A + pA
ApC	2.8 $\times$ 10^{-3}	9,600	A + pC
CpU	1.2 $\times$ 10^{-4}	25,600	C + pU

TABLE IV: Effect of Various Nucleotides and Polynucleotides on Hydrolysis of Nitrophenyl 5′-Thymidylate.[a]

Added Nucleotide	Concn (M × 10^3)	Hydrolysis of PN-pT; OD_{400} 5 min	10 min	15 min	Av Rel Act.
None		3.2	6.85	10.35	(100)
pC	1	2.1	4.9	7.6	73
d-pC	1	2.2	4.2	6.6	64
Cp	1	3.5	8.1	11.1	112
ApC	1	1.7	3.25	5.15	49
$U(pU)_6$	0.02	2.75	5.75	8.9	83
DNA	0.13	2.3	4.45	6.65	64

[a] Enzyme used: DEAE-cellulose preparation, 20 μg/ml under standard conditions. DNA was pneumococcal DNA, in mequiv of nucleotide.

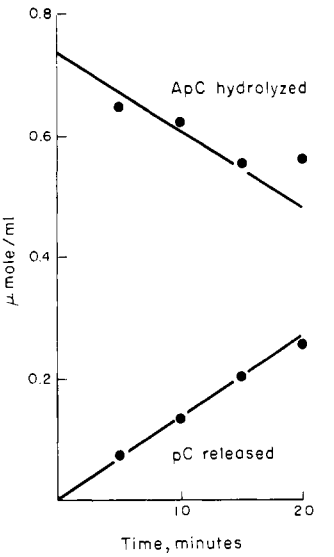

FIGURE 6: Correspondence of substrate disappearing with product formed. Quantitative paper chromatography as described in Materials and Methods.

from the paper; the spectral properties confirmed the identification by chromatographic mobility.

The total absorbance at peak wavelength permitted measurement of rates of hydrolysis. A typical time reaction course for substrate and product is shown in Figure 6; K_m and V_{max} were calculated using the double-reciprocal plot of Lineweaver and Burk (1934). Table III presents results for a number of experiments and substrates. With these ribonucleotides the enzyme shows the same requirements and other characteristics as with PN-pT, and the activity after DEAE-cellulose fractionation is likewise essentially linear with respect to protein concentration up to a concentration of 1–1.5 mg of protein/ml.

By paper chromatography the enzyme also gave complete cleavage of $U(pU)_6$ and $U(pU)_4$ to U and pU, and $A(pA)_4$ to A and pA but it was not possible to detect any oligonucleotide intermediate. $U(pU)_6$ gives a K_m of 2.2×10^{-4} M and V_{max} of 286 μmoles/hr per mg. There was suggestion of a substrate inhibition of hydrolysis by high concentrations of $Ap(A)_4$ and ApA. Under the conditions used for the oligonucleotides, active preparations did not release acid-soluble nucleotide from: RNA core (before or after being treated with alkaline phosphatase), virus f_2 RNA labeled with ^{32}P, yeast RNA labeled with ^{14}C, nor rat ribosomal ^3H-labeled RNA. The enzyme appears unable to cleave bis-(*p*-nitrophenyl) phosphate, adenosine 3′,5′-cyclic monophosphate, adenosine 2′,3′-cyclic monophosphate, cytidine 2′,3′-monophosphate, or UpUp, and does not release nitrophenol from *p*-nitrophenylthymidine 3′-phosphate.

Competition of Various Substrates and Products for the Enzyme. The enzymatic activity on PN-pT is reduced by several 5′-nucleotides in a manner at least roughly competitive at different concentrations. Among these potential enzyme products which are inhibitory are 5′-ribomononucleotides pU and pC, also 5′-deoxynucleotides d-pC, d-pU, and pT. Equivalent amounts of 3′-nucleotide Cp did not inhibit, but seemed rather to stimulate the hydrolysis. Some of these effects are shown in Table IV.

Several substrates which are hydrolyzed by the enzyme preparations also inhibit the hydrolysis of PN-pT under standard conditions. As shown in Table IV, this effect is exhibited by ribonucleotides ApC and $U(pU)_6$, and pneumococcal DNA.

Action of Enzyme on DNA. High molecular DNA from *E. coli* is hydrolyzed by the enzyme with release of low molecular products. The hydrolysis was investigated by gel filtration designed to show independently the nature of terminal and intermediate products.

Upon exposure to moderate levels of enzyme for 2–3 hr [^3H]thymidine-labeled DNA was rendered completely acid

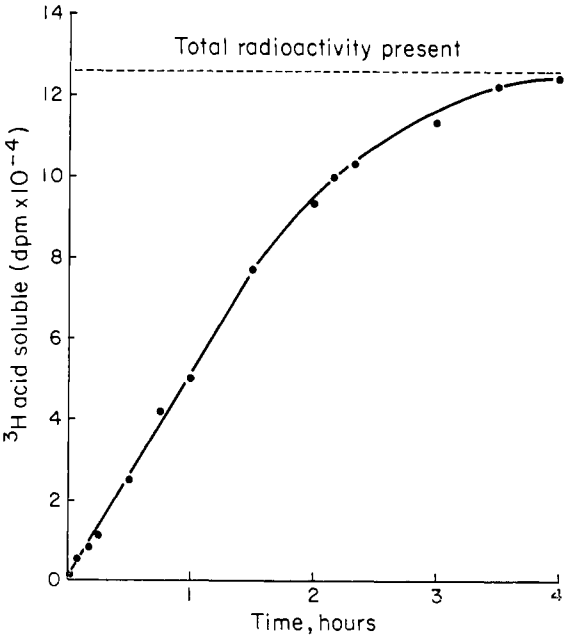

FIGURE 7: Solubilization of tritium-thymidine component from *E. coli* DNA by *L. acidophilus* exonuclease. Heated DNA (0.2 OD_{260} units total) was treated under standard reaction conditions.

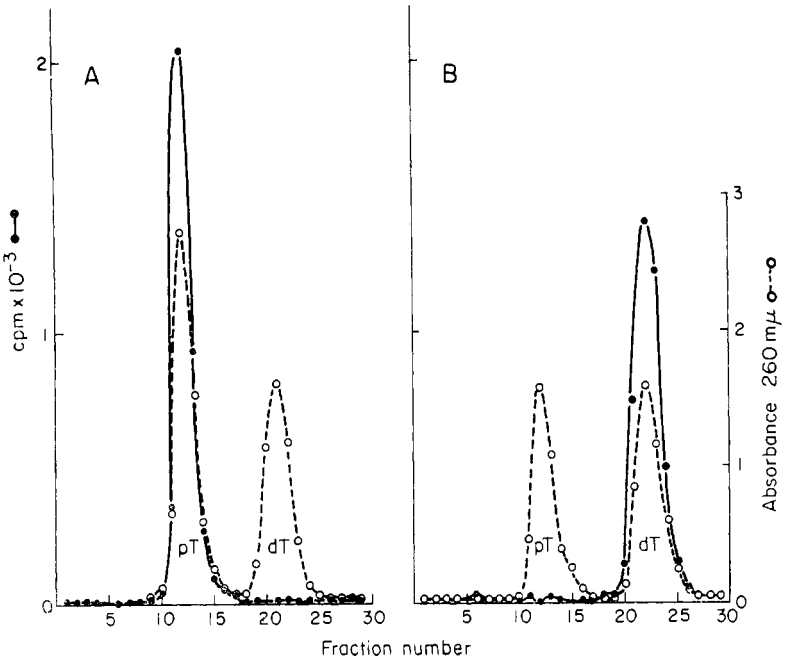

FIGURE 8: Gel filtration through Sephadex G-15 of completely hydrolyzed tritium-thymidine-labeled *E. coli* DNA. The labeled product (solid line, A) migrates with the added internal reference substance, 5′-thymidylic acid, the latter followed by ultraviolet absorbance. After treatment with *E. coli* alkaline phosphatase the labeled product is quantitatively converted into material (solid line, B) migrating with free thymidine. (The phosphatase used was demonstrated by Mr. Thomas Easton to be free of diesterase.)

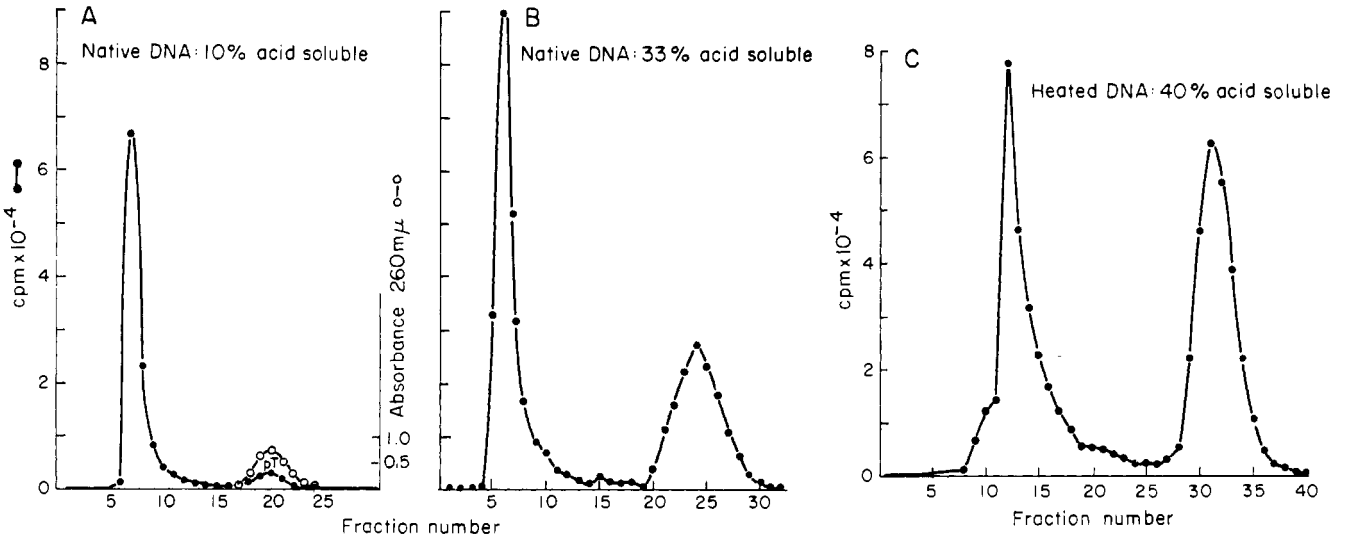

FIGURE 9: Mobility of early products of exonuclease hydrolysis of tritium-labeled *E. coli* DNA in Sephadex G-100, showing essential absence of labeled products of intermediate size. The first peak is high molecular material excluded by the gel, and the late peak coincides with that of thymidylate (supplied as ultraviolet-absorbing internal standard in run A).

soluble (Figure 7). The total hydrolysate passed through Sephadex G-15 gave the pattern indicated in Figure 8A, all radioactivity falling precisely at the position of the pT internal reference standard. The absence of higher nucleotides bearing thymidine is shown by the shift of label to the distinctly separate position of thymidine (dT, Figure 8B) after short treatment with bacterial alkaline phosphatase (monoesterase).

The liberated nucleotides appear to be of low molecular weight even from the beginning of enzymatic attack. As indicated in Figure 9 when only 10% of the label has been rendered acid soluble, that portion is recovered in a sharp single peak having the mobility of thymidylate. By contrast (Figure 10), a partial hydrolysis of pneumococcal DNA with pancreatic endonuclease gave intermediate material of notably larger size even when 65% had been solubilized. Once again the *Lactobacillus* enzyme showed no hydrolysis products of intermediate size even over the broad range (to 10^5 mol wt) resolved by Sephadex G-100. This comparison is essentially that developed by Birnboim (1966) for the classification of nucleases, and indicates that the enzyme is an exonuclease. Similar findings are obtained with both heated and unheated *E. coli* DNA, both giving, at early and late stages of hydrolysis, only products with the size and mobility of mononucleotides (Figure 9).

As with PN-pT and ApC, $MnCl_2$ (1×10^{-3} M) or $CaCl_2$ (1×10^{-3} M) are needed for the enzyme action; and EDTA in high concentration is inhibitory.

Viscosity. Pneumococcal DNA was treated with DNase I and *L. acidophilus* enzyme and its viscosity and perchloric acid solubility were followed. When DNase I has made 5% of the DNA acid soluble the relative viscosity has fallen to less than 1% of its initial value. When *L. acidophilus* enzyme has produced 30% acid-soluble material the viscosity has dropped only to 68% of the original value. These results are analogous to those of Lehman and Richardson (1964) obtained with *E. coli* exonucleases on poly d(A,T) copolymer. For these experiments the concentration of DNA was 100 μg/ml, that of DNase was 1 μg/ml. *L. acidophilus* enzyme was added at 40 μg/ml in TDM 7.9. For DNase $MgCl_2$ replaced $MnCl_2$.

Transformation. The biological activity of pneumococcal-transforming DNA is progressively destroyed by incubation with the purified 5′-diesterase fraction from *L. acidophilus*. For example, when genetically marked DNA (45 μg/ml) was treated with *Lactobacillus* enzyme (36 or 144 μg of protein per ml), the biological activity was reduced approximately 50 and 90%, respectively, in 30 min at 37°. The residual transforming activities at limiting concentration of DNA (Hotchkiss, 1966) were (in per cent of original values): streptomycin resistance, 47, 9; sulfanilamide resistance 50, 14, at the two enzyme concentrations. A duplex sulfonamide resistance locus (*sul*-ad) was reduced to 6% activity by the higher concentration.

Discussion

The *L. acidophilus* phosphoesterase described here displays a highly developed selectivity for hydrolysis of 5′-nucleotide esters. Activated with manganous salts and stabilized by a dithiol compound, it rapidly hydrolyzes the nitrophenol ester of 5′-thymidylate while having no action upon the 3′-diester or UpUp. It is therefore clearly distinct from the 3′-exonuclease discovered in the same organism by one of us and studied by Fiers and Khorana (1963).

When the 5′ enzyme acts upon ribodinucleoside monophosphates, XpY, the only products that can be detected are X and pY, the 5′-nucleotide. Equivalent hydrolysis can be observed on oligonucleotides up to at least the size of $U(pU)_6$, but several polyribonucleotides do not appear to be split. Among deoxynucleotides, $d\text{-}(pT)_4pC$ is hydrolyzed, and DNA, native and denatured, is attacked.

The clear evidence that those oligonucleotides which were tested, and DNA itself, inhibit the hydrolysis of the simple substrate, PN-pT, furnishes a strong indication that those actions which we have described are all attributable to a single catalytic entity, a 5′-nucleotide esterase. It should also be noted that all of these activities have the same requirements for cations and pH and are similarly influenced by EDTA.

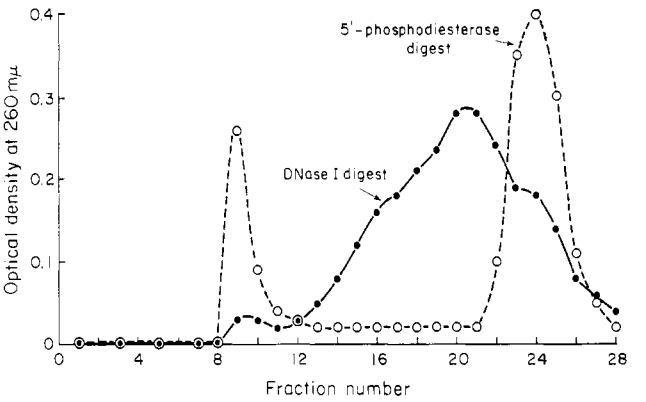

FIGURE 10: Gel filtration through Sephadex G-100 of partial hydrolysates of pneumococcal DNA (total products, indicated by OD_{260}). Hydrolysis by pancreatic endonuclease I (solid line) was stopped at 65% acid solubility. Hydrolysis by *L. acidophilus* exonuclease (dashed line) was stopped at 61% acid solubility.

The identification of this enzyme as a 5′-exonuclease is best supported by its action upon DNA. When pneumococcal DNA was degraded, biological activity and viscosity fell rather slowly, while even in the early stages of the attack, the ultraviolet-absorbing acid-soluble products appeared to be of the order of size of mononucleotides. It can be assumed that these are almost certainly the 5′-nucleotides. With tritium–thymidine-labeled *E. coli* DNA, the only labeled product detectable appeared to be the mononucleotide thymidylate at all stages of hydrolysis.

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